Int. J. Cancer: 65, 377–382 (1996)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

PULMONARY METASTASES NEUTRALIZATION AND TUMOR REJECTION BY *IN VIVO* ADMINISTRATION OF β GLUCAN AND BISPECIFIC ANTIBODY

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Bispecific antibody (BsAb) with specificity for tumor cell surface antigen and the CD3 molecule on T cells can redirect activated T cells to lyse tumor cells. Since the *ex vivo* expansion and activation of T cells is impractical and ineffective for treating established tumors, we tested whether the immune stimulant β glucan could *in situ*-activate T cells, which could secondarily be retargeted with BsAbs to lyse tumor cells. To test for tumor neutralization, C3H/HeN mice were injected i.v. with Cl-62 melanoma cells and immediately treated with i.p. β glucan and/or anti-CD3 (500A2) × anti-p97 (96.5) F(ab')$_2$ BsAb i.v. Pulmonary metastases were counted 14 days later. To test for tumor rejection and survival in a solid tumor model, mice were injected s.c. and i.p. with Cl-62 cells and 7 days later administered β glucan i.p. and/or F(ab')$_2$ BsAb i.v. In the neutralization model, there was a significant reduction in the number of metastases in the β glucan + BsAb group, as compared with controls, and with β glucan alone. In the established tumor model, β glucan + BsAb reduced the incidence of s.c. tumors as compared with control, with BsAb alone and with β glucan alone. It also prolonged survival of tumor-bearing mice compared with control, BsAb alone and β glucan alone. We conclude that T cells can be activated *in vivo* by β glucan and retargeted with F(ab')$_2$ BsAb.

Immune therapies represent an attractive option for the management of advanced malignancies resistant to conventional treatments. *In vitro*-activated and -expanded lymphocytes have been widely applied for the adoptive immunotherapy of cancer (West *et al.*, 1987; Rosenberg *et al.*, 1988), but unfortunately, protocols involving peripheral blood lymphocytes or tumor-infiltrating lymphocytes (TIL) have induced few clinical remissions and produced significant toxicity (Rosenberg *et al.*, 1988). As an alternative, adoptively transferred lymphocytes can be specifically retargeted using bispecific antibodies (BsAb).

BsAb with specificity for tumor cell surface antigens and specificity for the CD3 portion of the TCR/CD3 T-cell receptor complex effect tumor cell lysis by activating cytotoxic T lymphocytes and by physically linking the effector and tumor cell, thereby providing MHC-unrestricted target specificity (Perez *et al.*, 1986). We previously demonstrated that BsAb administered in conjunction with *in vitro*-activated lymphocytes lysed a variety of human colon cancer cell lines *in vitro* (Nelson *et al.*, 1990*a*) and neutralized human colon cancer cells growing *in vivo* in nude mice (Nelson *et al.*, 1991). Despite these favorable results, the eradication of established solid malignancies using adoptively transferred cells with BsAb has not been possible. Moreover, the *ex vivo* expansion of T lymphocytes is labor-intensive, impractical, expensive and likely to alter the cytotoxic and trafficking properties of lymphocytes, thereby reducing anti-tumor efficacies (Topalian *et al.*, 1987).

To bypass this *in vitro* activation step, we examined the possibility that *in vivo*-activated immune cells could secondarily be retargeted with BsAb to lyse tumor cells. β 1-3 glucans, which are potent biological response modifiers, were selected based on the fact that they activate multiple components of the cellular immune system. It has been shown *in vitro* that β glucans enhance the phagocytosis of syngeneic tumor cells by macrophages, activate and increase cytotoxicity of natural killer cells and increase the activity of killer T cells (Nanba *et al.*, 1987; Di Renzo *et al.*, 1991).

The following studies tested whether β glucans could produce *in vivo* T-cell activation and whether *in vivo*-activated T lymphocytes could be targeted to lyse tumor cells by BsAb. The resulting anti-tumor effects were evaluated in a syngeneic murine melanoma model in which both tumor neutralization and survival were examined. Therapeutic effects were further compared to those obtained with adoptively transferred, *in vitro*-activated lymphocytes retargeted with BsAb.

MATERIAL AND METHODS

Antibody and BsAb preparation

A monoclonal antibody (MAb) recognizing the p97 human melanoma antigen, 96.5, was kindly provided by Drs. K.E. and I. Hellström (Oncogene Corp., Seattle, WA). 500A2, a hamster anti-mouse MAb directed against the ε chain of murine CD3, was produced by i.p. growth of the 500A2 hybridoma (a gift from Dr. McKean, Rochester, MN) in nude mice, as previously described (Reid *et al.*, 1992*a*). 500A2 was purified from ascitic fluid using a protein A column, concentrated and sterile-filtered for tissue culture use. F(ab')$_2$ fragments of the MAbs 500A2 and 96.5 were produced by enzymatic digestion for 3 hr at 37°C, pH 4.5 and 4.0, respectively, using immobilized pepsin (Pierce, Rockford, IL) according to a previously described methodology (Parham, 1983). BsAb was prepared from F(ab')$_2$ fragments of 500A2 and 96.5 MAbs.

Antibodies were conjugated using a standard technique with N-succinimidyl-3-(2-pyridyldithio)-propionate forming covalent bonds between the 500A2 and 96.5, as described previously (Karpovsky *et al.*, 1984). Conjugated BsAb was separated from monomer using gel filtration and BsAb purity confirmed by SDS-PAGE gel electrophoresis. F(ab')$_2$ 500A2 × 96.5 (BsAb) was always administered by tail vein (i.v.) injection.

β glucan preparation

Barley β glucan (Sigma, St. Louis, MO) was suspended in PBS (400 μg/ml) and pressed 3 times through a syringe with a 27-gauge needle. Insoluble material was removed by centrifugation at 500 *g* for 5 min. β glucan was always administered as an i.p. injection.

Tumor cell line

Cl-62, a murine melanoma cell line syngeneic to C3H/HeN mice, is a clone of K1735 which has been transfected with the human gene coding for the p 97 antigen (Estin *et al.*, 1989). The Cl-62 cell line was kindly provided by Oncogene and maintained in culture as an adherent monolayer in RPMI 1640 medium supplemented with 10% heat inactivated FCS (GIBCO BRL, Grand Island, NY), 0.03% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium, CM). For inoculation, cells were harvested from a culture flask

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Received: July 31, 1995 and in revised form October 26, 1995.

with trypsin EDTA (GIBCO), washed and resuspended in CM.

Animal models—in vivo *methods*

Six- to 9-week-old female C3H/HeN mice were purchased from Charles River Laboratories (Wilmington, MA), and 6- to 9-week-old athymic nude mice (*nu/nu*) and BALB/c mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Animals were maintained in accordance with NIH animal care guidelines.

For tumor neutralization studies, Cl-62 tumor cells (10^5) were administered i.v. in 0.5 ml media to C3H/HeN mice on day 0, and animals were randomly assigned to treatment groups. Control animals received 0.5 ml of PBS, i.p., from day 0 to day 10. β glucan was administered in 0.5 ml PBS, i.p., beginning 10 min after Cl-62 injections and then daily for 10 consecutive days. 500A2 $F(ab')_2 \times 96.5$ $F(ab')_2$ BsAb was given at the dose of 5 μg in 0.5 ml PBS, i.v., at day 0. When β glucan and BsAb treatments were combined, the same doses and schedules were used as in the single reagent regimen. *In vitro*, culture-activated splenocytes (4×10^7 cells) were injected i.v. in 0.5 ml media on day 0. Mice were killed 14 days after Cl-62 tumor cell injection by ether inhalation. Lungs were injected with india ink *via* the trachea, then harvested and destained with Fekete's solution (Wexler, 1966). The 5 lobes of the lungs were separated and the number of metastases per mouse counted by 2 independent observers under a dissecting microscope. Lungs with more than 250 metastases were reported as 250.

For established tumor studies, to evaluate for survival, tumor regression and intra-tumor effects, C3H/HeN mice were injected s.c. and i.p. with 4×10^6 Cl-62 cells at each site. Previous studies in our laboratory have shown that this model produces microscopic tumors at the site of injection by day 5 and macroscopic tumors by days 10–15. Treatments were, therefore, initiated 7 days after tumor cells were injected. Control animals received 0.5 ml of PBS, i.p., days 7–16; β glucan was given at a dose of 200 μg i.p. in 0.5 ml PBS, days 7–16; 500A2 × 96.5 $F(ab')_2$ BsAb was injected i.v. *via* a tail vein at a dose of 5 μg on days 8 and 18. When β glucan and BsAb treatments were combined, doses and schedules were the same as for the single reagent regimen. Subcutaneous tumors were measured every 2 days beginning 10 days after tumor cell injecetions, and animals were followed until death or 120 days.

Splenocyte, lymph node cell and TIL preparations

Spleens and inguinal lymph nodes were harvested from C3H/HeN mice under sterile conditions. Cells were expressed and washed twice in CM supplemented with 1 mM sodium pyruvate (GIBCO), 0.1 mM non-essential amino acids (GIBCO), 10 mM HEPES (Sigma), 0.01 mM 2-mercaptoethanol (Sigma) and 40 mg/l gentamicin (ESI Pharmaceutical, Cherry Hill, NJ). Single-cell suspensions (10^6/ml) were prepared for *in vitro* studies.

For therapeutic use, splenocytes were cultured 5 days in supplemented media (100 U/ml rIL-2, a gift from Hoffmann-La Roche, Nutley, NJ) and 0.4 mg/ml 500A2 at 5% CO_2 at 37°C. Excess 500A2 was eliminated by washing and replating in medium with IL-2 alone 24 hr before use.

TIL were obtained from s.c. tumors of Cl-62 measuring 0.5–1 cm in C3H/HeN mice. Tumors were harvested aseptically, minced into small fragments and digested for 4 hr in 40 ml RPMI 1640 containing 40 mg collagenase, 4 mg DNase and 100 UI hyaluronidase (all from Sigma). Resulting cell suspensions were prepared (10^6/ml) for FACS analysis or proliferation assays.

In Vitro *methods*

Lymph node cells, splenocytes and TIL were analyzed on a FACS IV flow cytometer (Becton-Dickinson, Mountain View, CA). Fluoresceine isothiocyanate- and phycoerythrin-labeled MAbs for murine markers included: Lyt-2 (cytotoxic T cells), L3T4 (T helper cells) (both from Becton Dickinson, CD3ε (pan T cell) and CD25 (IL-2 receptor, [IL-2R]) (both from PharMingen, San Diego, CA). Isotype-matched controls were used in all experiments.

Proliferation studies were performed by co-culturing 10^5 splenocytes, lymph node cells or TIL from control or treated mice with either media, rIL-2 (10 U/ml) or 2×10^5 of irradiated (3,300 rads) BALB/c splenocytes for 72 hr in 96-well, U-bottomed microtiter plates at 37°C. Proliferation was determined by [^{3}H] thymidine uptake (1 μCi/well for 24 hr), and all assays were performed in triplicate.

Cytotoxicity was measured in a standard 4-hr ^{51}Cr-release assay as previously described (Nelson *et al.*, 1990*a*). Each assay was performed in triplicate, with lytic effect expressed as percentage of specific ^{51}Cr-release and <20% background lysis in all reported assays.

Statistics

For tumor neutralization studies, results were compared using a Mann-Whitney rank sum test, assuming a non-Gaussian distribution for the mean number of metastases. Survival curves were compared with log-rank tests. Probability values below 0.05 were considered significant.

RESULTS

Effects of β glucan on in vivo *T-cell activation and tumor neutralization*

The potential for β glucan alone to activate T cells *in vivo* was tested by examining IL-2R expression on T cells of mice treated for 10 consecutive days with either PBS (control) or β glucan (200 μg/day). Flow cytometric analysis demonstrated a consistent increase of IL-2R expression on lymph node cells from 15% ± 4% (control) to 26% ± 10% (β glucan-treated) and on splenocytes from 32% ± 8% (control) to 40% ± 13% (β glucan-treated). The *in vivo* effects of β glucan on T-cell activation were further tested using proliferation assays. Treatment with β glucan (200 μg/day for 10 consecutive days) only moderately enhanced the spontaneous proliferative response of splenocytes and lymph node cells *vs.* control. The proliferative response to allogeneic cells or rIL2 of splenocytes and lymph node cells from β glucan-treated animals was also consistently enhanced (Table I).

In vivo T-cell activation with β glucan did not result in T-cell depletion as the percentages of $CD3^+$ lymph node cells were 70% and 76% in the control and treated groups, respectively,

TABLE I – *IN VIVO* EFFECTS OF β GLUCAN ON LYMPHOCYTE PROLIFERATION[1]

	In vivo treatment group[2]	
	Control	β glucan
Splenocytes		
Media alone	2,733	5,902
Allogeneic cells (BALB/c)	22,187	32,760
rIL2 (10 U/ml)	23,606	39,226
Lymph node cells		
Media alone	1,702	4,071
Allogeneic cells (BALB/c)	30,756	36,403
rIL2 (10 U/ml)	34,663	43,690

[1]Proliferative responses to media alone, allogeneic cells and rIL2 in control mice (PBS i.p. for 10 days) and in β glucan–treated mice (β glucan 200 μg i.p. for 10 consecutive days) were measured at 72 hr.–[2]Results are expressed as mean cpm.

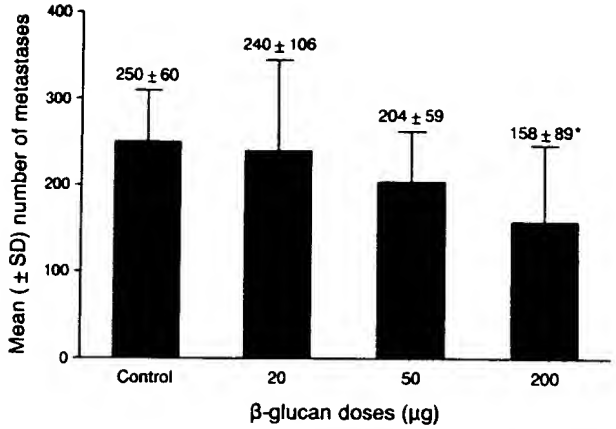

FIGURE 1 – Dose-response effects of β glucan on number of pulmonary metastases. C3H/HeN mice (5/group) were injected i.v. with 10^5 Cl-62 melanoma cells and then β glucan was administered at a dose of 0, 20, 50 or 200 μg every day for 10 consecutive days. Mean pulmonary metastases ± SD were counted 14 days after Cl-62 cells were injected. $^*p < 0.05$ *vs.* control (Mann-Whitney).

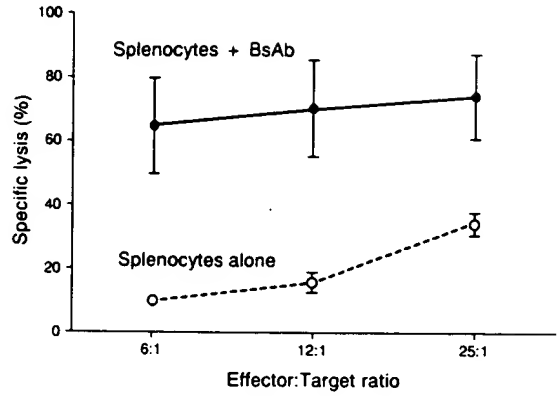

FIGURE 2 – BsAb retargets β glucan *in vivo*–activated splenocytes to lyse Cl-62 tumor cells. Splenocytes harvested from C3H/HeN mice treated with β glucan (200 μg for 10 days) were cultured for 5 days and tested for lysis of radiolabeled Cl-62 cells with or without 500A2 × 96.5 $F(ab')_2$ BsAb (20 ng/well) in standard 4 hr chromium-release assays at 3 effector:target ratios.

and the percentages of $CD3^+$ splenocyte were 14% and 20% in the control and treated groups, respectively.

To assess the tumor-neutralizing effects of β glucan, C3H/HeN mice were treated immediately after an i.v. injection of 10^5 Cl-62 cells with 0, 20, 50 or 200 μg of β glucan for 10 days. The mean number of pulmonary metastases counted 14 days after the tumor cell injection is shown in Figure 1. Doses of 200 μg significantly decreased the number of metastases compared with the other groups ($p < 0.05$). No toxic effects were seen.

Effects of combined treatment with β glucan and BsAb: tumor neutralization studies

To confirm that *in vivo* β glucan-activated T cells could be redirected by 500A2 × 96.5 $F(ab')_2$ BsAb to lyse Cl-62 tumor cells, splenocytes from β glucan-treated animals were tested in 4 hr cytotoxicity assays. Splenocytes harvested from C3H/HeN mice treated with β glucan, 200 μg for 10 days, were cultured for 5 days and then tested for lysis of radiolabeled Cl-62. No significant cytotoxicity was detected for splenocytes alone, but the addition of BsAb enhanced lysis of the melanoma cells for all effector:target ratios tested (Fig. 2).

The ability for β glucan-activated lymphocytes to be retargeted by BsAb to achieve tumor neutralization *in vivo* was then assessed by comparing the number of pulmonary metastases after treatment with saline control, β glucan alone (200 μg), BsAb alone (5 μg) and β glucan (200 μg) + BsAb (5 μg), as described in "Material and Methods". As shown in Figure 3, the mean number of metastases was significantly lower for the β glucan + BsAb-treated group *vs.* control ($p < 0.01$) and *vs.* β glucan alone ($p < 0.02$).

To compare the anti-tumor effects of BsAb-retargeted, β glucan-activated lymphocytes and the effects of BsAb-retargeted, adoptively transferred cells, animals were treated 10 min after i.v. injection of 10^5 Cl-62 cells, with either saline (control), β glucan (200 μg), β glucan (200 μg) + BsAb (5 μg) or *in vitro*-activated splenocytes (4×10^7) + BsAb (100 μg). Both the *in vitro*-activated splenocytes and β glucan, when combined with BsAb, reduced the number of metastases compared with control. However, combined treatment with β glucan + BsAb was significantly more effective than BsAb + adoptively transferred, *in vitro*-activated splenocytes ($p < 0.05$; Fig. 4).

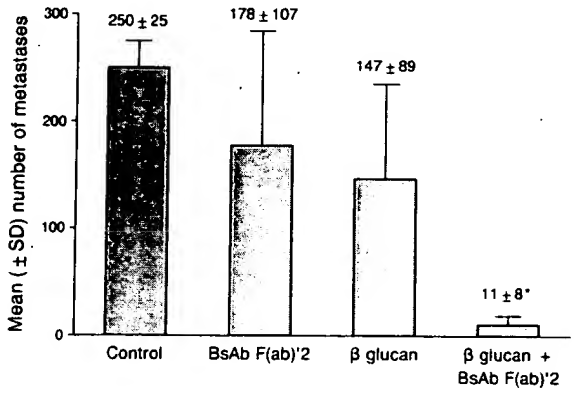

FIGURE 3 – Tumor neutralization following treatment with β glucan and BsAb alone and combined. Following i.v. injection of 10^5 Cl-62 tumor cells, mice (5/group) were treated with 0.5 ml PBS for 10 days (control), 200 μg of β glucan for 10 days, 5 μg of 500A2 × 96.5 $F(ab')_2$ BsAb on day 0 or β glucan (200 μg for 10 days) + BsAb (5 μg on day 0). Mean pulmonary metastases ± SD were counted 14 days after Cl-62 injections. $^*p < 0.01$ *vs.* control, $p < 0.02$ *vs.* β glucan alone (Mann-Whitney).

Intra-tumor effects of combined treatment with β glucan and BsAb: established tumor model studies

The high therapeutic potency of BsAb-retargeted, β glucan *in vivo*-activated cells prompted further testing in an established tumor model. To assess the effects of combined treatment with β glucan + BsAb on T-cell sub-populations, FACS analyses were performed on splenocytes, lymph node cells and TIL from control mice (PBS), β glucan-treated mice (200 μg) and mice treated with β glucan (200 μg) + 500A2 $F(ab')_2$ × 96.5 $F(ab')_2$ (5 μg). Results are shown Table II. The proportion of $CD3^+$ cells in TIL was doubled after combined treatment with β glucan + BsAb compared with β glucan alone or control. After combined treatment, the percentage of $CD8^+$ T cells in TIL was augmented and the ratio of $CD4^+$/$CD8^+$ cells inversed. Combined treatment, as described above, also increased the proliferative response of TIL to allogeneic cells (Table III).

The *in vivo* effects of β glucan and BsAb, both together and alone, were tested in an established tumor model, where Cl-62 tumor cells were injected i.p. and s.c. 7 days before treatments

were administered. Measurable s.c. tumors developed in all (10/10) animals treated with saline (control). Compared to controls, there was no reduction in the number of tumors in mice treated with BsAb alone (9/10 with tumors), but there was a significant reduction in the β glucan alone group (6/10, $p < 0.05$) and in the β glucan + BsAb group (2/10, $p < 0.01$). The incidence of tumors in the β glucan + BsAb group was significantly lower than in the β glucan alone and the BsAb alone groups (both $p < 0.05$).

Differences noted in the s.c. growth of established tumors translated into differences in survival (Fig. 5). Compared to a saline control group, BsAb alone had no effect on survival ($p = 0.31$), but both β glucan alone and β glucan + BsAb significantly prolonged survival ($p = 0.047$ and $p = 0.0001$, respectively). Survival was significantly prolonged in the β glucan + BsAb group compared with the BsAb alone ($p = 0.005$) and the β glucan alone ($p = 0.5$) groups.

DISCUSSION

Immune therapies for solid malignancies have focused on non-specific stimulation of the immune system or the adoptive transfer of immune cells. Non-specific stimulation with agents such as the interferons, IL-2 and LAK cells have been associated with considerable toxicity owing to the high doses required to produce anti-tumor responses (Rosenberg *et al.*, 1988; Lotze *et al.*, 1985). Adoptive transfer of cytotoxic T lymphocytes or TIL, which exhibit potent lytic activities and target specificity, has been limited by the complexity of the *in vitro* activation procedures and the inability of transferred cells to traffic to tumor sites. To overcome these limitations, we have induced *in vivo* T-cell activation using a non-toxic agent to provide a population of activated T cells that could be retargeted with BsAb to lyse tumor cells.

Because of the lack of availability of MAbs specific for murine tumors, many animal studies have used xenogeneic models in which human tumors were transplanted into immunodeficient mice (Nelson *et al.*, 1991). In the present experiments, the use of an immunocompetent mouse strain and a syngeneic tumor provided a more realistic model for assessing

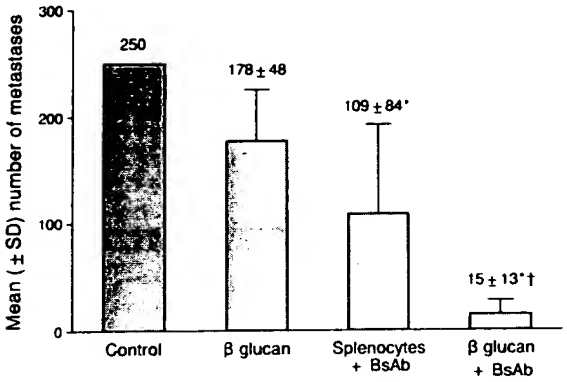

FIGURE 4 – Comparative tumor neutralization from BsAb combined with *in vivo* β glucan activation *vs. in vitro* activation with adoptive transfer. Mice (5/group) received 10^5 Cl-62 melanoma cells and immediate treatment with β glucan (200 μg for 10 days), 500A2 × 96.5 $F(ab')_2$ BsAb (5 μg, day 0), β glucan + $F(ab')_2$ BsAb (same doses) or *in vitro*–activated splenocytes (4×10^7 i.v. day 0) + $F(ab')_2$ BsAb (100 μg, day 0). Mean pulmonary metastases ± SD were counted 14 days after Cl-62 injections. *$p < 0.05$ *vs.* control (Mann-Whitney). †$p < 0.05$ *vs.* splenocytes + BsAb (Mann-Whitney).

TABLE III - PROLIFERATIVE RESPONSES

	In vivo treatment group[2]		
	Control	β glucan	β glucan + BsAb
Splenocytes	13,961	36,414	36,745
TIL	7,738	6,844	12,847

[1]Proliferative responses of splenocytes and TIL to allogeneic BALB/c cells in control mice (PBS i.p. days 7–16), β glucan–treated mice (200 μg i.p. days 7–16) or β glucan + BsAb–treated mice (β glucan 200 μg i.p. days 7–16, 500A $F(ab')_2 \times 96.5$ $F(ab')_2$ BsAb 5 μg i.v. on days 8 and 18) were measured at 72 hr.–[2]Results are expressed as mean cpm.

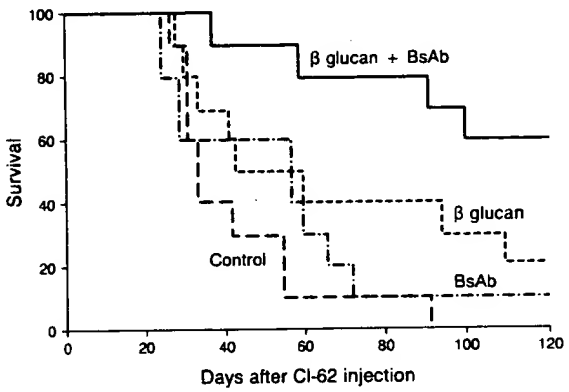

FIGURE 5 – Effects of β glucan and BsAb on survival in an established tumor model. C3H/HeN mice (10/group) were treated 7 days after injection of 4×10^6 Cl-62 melanoma cells i.p. and s.c. with either saline, β glucan (200 μg for 10 days), 500A2 × 96.5 $F(ab')_2$ BsAb (5 μg on days 8 and 18) or β glucan + BsAb, using the same doses and schedules.

TABLE II - FLOW CYTOMETRIC ANALYSIS OF INTRA-TUMOR EFFECTS OF β GLUCAN AND BsAb

	In vivo treatment group[2]		
	Control	β glucan	β glucan + BsAb
Lymph node cells			
$CD3^+$	70 ± 10%	76 ± 3%	75 ± 9%
$CD4^+/CD8^+$	51 ± 6%/18 ± 3%	50 ± 10%/25 ± 8%	53 ± 4%/21 ± 6%
TILs			
$CD3^+$	16 ± 8%	18 ± 3%	35 ± 4%
$CD4^+/CD8^+$	54 ± 5%/19 ± 2%	56 ± 1%/23 ± 8%	24 ± 6%/45 ± 5%

[1]T-cell populations of lymph node cells and tumor-infiltrating lymphocytes were harvested from control mice (PBS i.p. days 7–16), β glucan–treated mice (200 μg i.p. days 7–16) and β glucan + BsAb–treated mice (β glucan 200 μg i.p. days 7–16, 500A $F(ab')_2 \times 96.5$ $F(ab')_2$ BsAb 5 μg i.v. on days 8 and 18).–[2]Results are expressed as percent positive cells (±SD) after subtraction of background levels of staining with control MAb.

immune cell responses and measuring pre-clinical anti-tumor effects. In this model, we previously demonstrated that anti-tumor effects of BsAb alone were weak and limited due to T-cell depletion effects induced by doses of BsAb greater than 50 μg (Penna *et al.*, 1994). BsAb doses of 5 μg were therefore used. An $F(ab')_2$ BsAb was preferred for these *in vivo* studies since these reagents produce optimal tumor:tissue ratios (Nelson *et al.*, 1990*b*).

Intraperitoneal administration of β glucan produced some degree of T-cell activation, as demonstrated by increased IL-2R expression and enhanced proliferative responses of lymph node cells and splenocytes in the presence of IL-2 or allogeneic cells. Based on the work of others, it seems unlikely that β glucan produces direct effects on T cells. It is known that β glucan produces mononuclear phagocyte activation *in vitro* and *in vivo* (Rasmussen and Seljelid, 1991). Since macrophages stimulated with β 1-3 glucan release IL-1 and the autogenous production of IL-1 is enhanced by β glucan in a small and prolonged fashion in both peritoneal fluid and peripheral blood (Rasmussen and Seljelid, 1991) and since IL-1 is a potent T-cell activator, it is postulated that T-cell activation induced by β glucan is related to IL-1 production.

In the pulmonary metastasis model, β glucan alone exhibited anti-tumor activities. Administration of β glucan has been found by others to potentiate the function of natural killer cells, which exhibit potent anti-tumor activities (Di Renzo *et al.*, 1991). β glucans also activate macrophages, which have been shown to be important in inhibiting the metastases of experimental tumors (Fidler *et al.*, 1987). Indirect T-cell activation could also explain anti-tumor activities of β glucan since T-cell activation with low doses of anti-CD3 MAb has been shown to result in significant anti-tumor effects in allogeneic tumor models (Ellenhorn *et al.*, 1988) and since *in vivo* activation of T cells with staphylococcal enterotoxin-B can facilitate tumor rejection in immunocompetent animals (Newell *et al.*, 1991).

Combined treatment with β glucan and BsAb was more effective than β glucan alone for pulmonary metastasis neutralization. Although BsAb alone can activate T cells by binding the $CD3^+$ portion of the T cell, the need for adding T-cell stimulation for effective BsAb treatment of tumor has been demonstrated (Weiner, 1992). These combined effects cannot be explained on the basis of antibody-dependent cellular cytotoxicity as a mechanism since the BsAb used, made with 2 covalently linked $F(ab')_2$ fragments, lacked the Fc fragments required for antibody-dependent cellular cytotoxicity. The possible role of T-cell activation mediated by the anti-CD3 portion of the BsAb has been examined, but the 500A2 MAb component of the BsAb has not shown any beneficial effect when compared to β glucan alone in the pulmonary metastasis model (data not shown). It is proposed, therefore, that BsAb effectively retargeted T cells activated *in vivo* with β glucan, as suggested by *in vitro* studies of cytotoxicity.

We next compared the effectiveness of this *in vivo* combined treatment with the more conventional approach of *in vitro*-activated, adoptively transferred lymphocytes retargeted with BsAb. An adoptive therapy treatment regimen likely to provide the maximal anti-tumor effects was chosen from previous studies, and a lymphocyte:tumor cell ratio of 400:1 was accomplished (Reid *et al.*, 1992*b*). In this neutralization model, trafficking of adoptively transferred cells to the site of tumor, which constitutes the major obstacle to adoptive therapy, is minimized. Despite this advantage, BsAb retargeting of β glucan *in vivo*-activated T cells was still found to be significantly more effective than BsAb retargeting of adoptively transferred splenocytes.

This anti-tumor efficacy led us to test β glucan + BsAb in the treatment of established tumors, where it is known that adoptive therapy is limited by trafficking inefficiencies. Combined treatment with β glucan and BsAb increased the number of $CD3^+$, and particularly $CD8^+$ cells, in TIL. Whether this is due to the primary binding of T cells to tumor cells *via* the BsAb or to the secondary effects of cytokine release in the tumor micro-environment needs further evaluation. After combined treatment, TIL also showed increased proliferative response to allogeneic cells. All together, these findings may account for the decreased tumor incidence and the prolonged survival observed after treatment of tumor-bearing animals with β glucan + BsAb.

In summary, the above studies demonstrate that i.p. administration of β glucan activates T cells that can be redirected by BsAb to lyse relevant tumor cells. β glucan alone has limited anti-tumor activity, with significant effects only at high doses, yet when β glucan is combined with BsAb, pulmonary metastases can be effectively neutralized. Not only is BsAb + β glucan superior to BsAb + *ex vivo*-activated, adoptively transferred lymphocytes in the neutralization model, but more importantly, β glucan + BsAb induced tumor regression and prolonged survival in tumor-bearing immunocompetent mice. Since β 1-3 glucans have already been used in human trials and their lack of toxicity is well established, these experimental results provide the rationale for testing combined treatment with β glucan and BsAb in humans with cancer refractory to standard therapeutic regimens.

ACKNOWLEDGEMENTS

C.P. was supported by a grant from the Société Nationale Française de Gastroentérologie. H.N. was supported by a Career Development Award (American Cancer Society).

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